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Host-specific cues cause differential attractiveness of Kenyan men to the African malaria vector *Anopheles gambiae*

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Abstract

Background: Many studies have suggested that variability in the attractiveness of humans to host-seeking mosquitoes is caused by differences in the make-up of body emanations, and olfactory signals in particular. Most investigations have either been laboratory-based, utilising odour obtained from sections of the body, or have been done in the field with sampling methods that do not discriminate between visual, physical and chemical cues of the host. Accordingly, evidence for differential attractiveness based on body emanations remains sparse in spite of the far-reaching epidemiological implications of this phenomenon.

Methods: A new three-port olfactometer that accommodates complete human beings as sources of host-seeking stimuli was used to study behavioural responses of *Anopheles gambiae* Giles *sensu stricto* (hereafter *An. gambiae*) under semi-field conditions in western Kenya. Differential attractiveness of nine male Kenyans was assessed by simultaneously exposing the mosquitoes to (a mixture of) total body emanations of 3 people occupying separate tents. Controls (empty tents) were included and the effect of residual odours following tent occupation was also examined.

Results: Trap catches increased significantly ($P < 0.001$) when a tent was occupied. Based on 'competition' experiments, the nine persons were classified into least, medium and most attractive groups. There was no significant interaction between person and trap ($P = 0.302$) or person and test period ($P = 0.223$). Presence ($P < 0.001$) or absence ($P = 0.949$) of significant differences in the number of mosquitoes caught per trap when tents were simultaneously occupied by one person in each or left empty, respectively, demonstrated that residual odours following tent occupation did not affect behavioural responses of the mosquitoes.

Conclusion: We provide evidence that in the vicinity of humans, when exposed to a blend of physical and olfactory signals from more than one host, *An. gambiae* can effectively and consistently express host-selection behaviour that results in non-random biting.

Background

Although there is evidence that some humans are more attractive to host-seeking African malaria mosquitoes than others [1–3] the reasons for this variability are not clearly understood [4]. Various studies have implicated some *Anopheles* species as preferring to feed on adults rather than children [5,6] and on men rather than women [6]. The preference for feeding on adults rather than children by *An. gambiae s.l.* [7–9] has been attributed to size [10] and surface area and weight [11]. In contrast, random feeding irrespective of age [12,13] and sex [10,11] by this mosquito and age, height and weight among the Asian *An. punctulatus* complex species has also been reported [14]. Some studies have shown that pregnancy [15,16], parasite infection [17] and ABO blood group type [18–20] influence attractiveness of humans to mosquitoes while others have refuted the influence of these host conditions [14,21,22].

In two of the studies reported above [10,15] it was postulated that variability in human attractiveness to mosquitoes is related to the amount of exhaled breath and volatile substances released from the skin. These hypotheses are plausible for at least two reasons. First, mammalian semiochemical blends are complex and include indicators of sex, general health condition, age, reproductive status and diet [23]. Second, mosquitoes are attracted to human hosts by responding to body emanations [24,25] and body odour is responsible for > 90% of the attractiveness of humans to *An. gambiae* [26].

It has been hypothesised that body odour can influence the choice by mosquitoes of a particular individual upon encountering a group of human hosts [2]. Odours emanating from hands and forearms have been shown to cause individual differences of attractiveness to *An. stephensi* Liston [27]. *Aedes aegypti* L. has been observed to probe on the forearms of certain individuals more than on those of others [28]. Significant differences in the response of *Ae. aegypti* and *An. quadrimaculatus* May to substances collected from hands of different human individuals has also been reported [29]. Although these studies provide an alternative reason for variability in human attractiveness to mosquitoes, the odour emanating from hands and forearms is not representative of that emanating from the entire body [30]. Therefore, we cannot conclude from these studies that complete body emanations are associated with differences in attractiveness of humans to mosquitoes as substances from different body parts of one individual can elicit significant variations in behavioural responses of mosquitoes [29].

Field studies in Burkina Faso demonstrated that differences in human attractiveness to *An. gambiae* and *An. funestus* Giles can be associated with olfactory cues released by the body, in particular carbon dioxide from expired breath [31]. These studies utilised odour-baited entry traps (OBETs), which served to separate olfactory cues from visual features of the host and its convective or radiant heat [32]. The OBETs have the inherent disadvantage of increasing experimental variance because of the varying experimental conditions of the tests [33]. In the current study we investigated the effects of complete body emanations including body odour, heat and moisture on differential attractiveness of humans to *An. gambiae*. We sought to (i) develop an olfactometer that accommodates humans as sources of host-seeking stimuli and use it to test whether complete body emanations are associated with variability in attractiveness to mosquitoes (ii) assess whether attractiveness of humans to mosquitoes can be ranked based on behavioural responses towards complete body emanations and (iii) find out whether mosquitoes can be attracted to tents which have been previously occupied by humans in response to residual stimuli. Unlike any of the studies reported above, our design is unique in the sense that it examines mosquito responses to *blends* of odours from several humans, which mimics what mosquitoes experience when entering a bedroom in a common African setting.

Methods

Mosquitoes

Experiments were conducted using laboratory-reared *An. gambiae* mosquitoes established from specimens collected in Njage village, 70 km from Ifakara, south-east Tanzania, in 1996. The mosquitoes were reared at ambient temperature and humidity at insectaries of the Mbita Point Research and Training Centre of the International Centre of Insect Physiology and Ecology (ICIPE). Mbita Point is located on the southern shore of the Winam gulf of Lake Victoria in Nyanza Province, Kenya (00°25' S, 34°13' E). Adult female mosquitoes were routinely offered a human arm to feed upon. Larvae were fed on Tetramin® fish food three times per day. The larvae were reared in plastic pans (25 × 20 × 14 cm) filled with fresh water from Lake Victoria to a depth of 3 cm. Trays contained 100 – 150 larvae. Pupae were collected daily and kept in mesh-covered cages (30 × 30 × 30 cm) containing 6% glucose solution on filter-paper wicks. Adult females were used for experiments when 4 – 8 days old and had no prior access to a blood meal.

Olfactometer bioassay

Experiments were conducted using an olfactometer designed to accommodate humans as sources of host-seeking stimuli (Figure 1). The set-up consisted of three tents connected to a central collecting system by polyvinyl chloride (PVC) pipes. The collecting system consisted of a choice chamber and three trap chambers. The choice chamber opened into mesh-covered collecting cages (12 ×

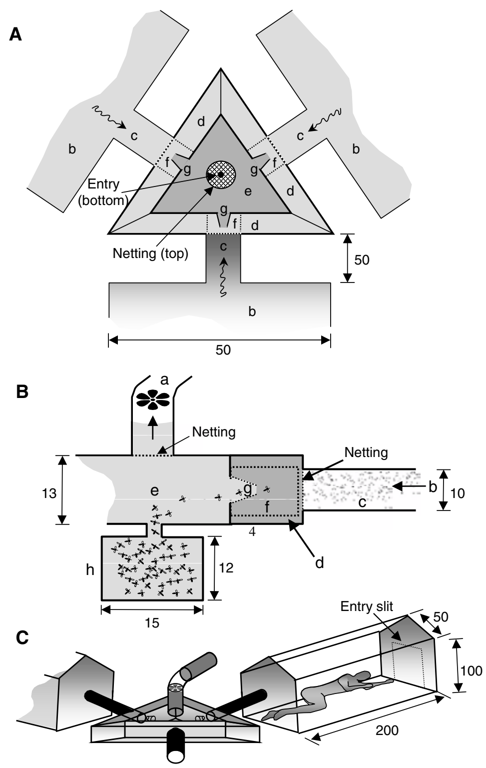

Figure 1
Top (A), cross-sectional (B) and three-dimensional (C) views of the experimental setup. The fan (a) drew air (~130 L/min/tent) from the three tents (b) to the outside environment via PVC pipes (c), trap chambers (d) and central choice chamber (e). Each trap chamber contained a collecting cage (f) into which an exit trap opened (g). The fan pipe and release cup (h) were fitted to the top and bottom of the choice chamber, respectively. Diagrams are not shown to scale; all dimensions are in centimeters.

12 × 12 cm), placed inside the trap chambers, through 8-cm (mouth diameter) funnels made of netting mesh. The collecting system, pipes and tents were covered with opaque polythene sheets to exclude visual cues. Tent-ends proximal to the collecting system had sleeves in which the pipes were fitted. Pipe-ends opening into trap chambers plus the fan-pipe, at its point of connection on the top lid, were covered with panels of mosquito netting. A CDC light-trap fan was used to draw air (~130 L/min/tent) from all three tents into the central chamber. The system was housed inside a large screen house (11.4 × 7.1 m) lined with mosquito netting along its walls. The roof of the screen house was covered with glass and the sides with mesh (density 90%). A layer of reed mats was placed beneath the roof so as to lower temperatures. The fan pipe was extended through the screen house wall, and delivered odour-laden air from the olfactometer to the outside of the screen house.

Mosquito behaviour in the absence of host emanations
Releasing mosquitoes and noting the number caught per trap in the absence of humans in any of the tents tested the symmetry and neutrality of the olfactometer. Tests were conducted on six experimental nights between 20.00 – 21.00, 22.00 – 23.00 and 24.00 – 01.00 hours. About 100 mosquitoes, starved for 6 hours, were released during each test period.

Mosquito behaviour in the presence of human emanations
Nine male Kenyans, aged 18–22 years, volunteered to participate in experiments designed to establish whether differential attractiveness to mosquitoes can be revealed on the basis of total body emanations. They were designated as P_1 – P_9. No special criteria were used to select the volunteers. Three persons were compared for their attractiveness to the mosquitoes within a single experiment. The relative attractiveness of each person was compared to that of P_1 at least three times. Three experiments were carried out per night between 20.00 – 21.00, 22.00 – 23.00 and 24.00 – 01.00 hours. Either 50 or 100 female mosquitoes, starved for 6 hours, were used in each experiment.

The number of mosquitoes caught in each trap chamber was considered as having been attracted by the person sleeping in the adjacent tent. Persons were alternated between tents, ensuring completion of a Latin square design on each experimental night. The persons shifted with their bedding material. The participants (i) lay with their feet proximal to the collecting system (ii) bathed with non-perfumed soap one hour prior to the first experimental period (iii) did not use perfumes or deodorants during the recruitment period (iv) only wore a pair of short trousers while inside the tents and (v) did not cover themselves with sheets or blankets during the experiments. Smears of their blood were examined microscopically for the presence of malaria parasites. Diets of the test persons were not controlled except prohibiting them from consuming alcohol, which has recently been shown to affect the attractiveness of humans to *Ae. albopictus* Skuse mosquitoes [34]. However, Ugali (a type of pasta made from maize meal), served with green vegetables (mainly collards (*Brassica oleracea*)), is the typical diet of the people of Mbita Point.

Effects of residual odour on mosquito behaviour
The effects of residual human emanations on mosquito responses were studied by releasing mosquitoes when all 3 tents of the olfactometer were occupied by one human subject in each (between 20.00 – 20.45 hours), followed by a second release when the tents were no longer occupied (21.30 – 22.15 hours). The persons who participated

in these experiments were P_2, P_5 and P_6, with only their bedding material being present in the tents during the second test period. Each experiment utilised 100, 6-hour starved female mosquitoes. The human subjects were not shifted between tents.

Statistical analysis

The number of mosquitoes caught in the presence and absence of host-seeking stimuli with respect to trap, person and test period were analysed by log-linear modelling [35]. This allowed for differences between traps, test periods and persons. A model of the form log (μ_{ijk}) = $E_i + T_j + P_k$, where E_i, T_j and P_k are the parameter estimates for experimental period *i*, trap *j* and person *k*, respectively, was fitted. Thus, the proportion of mosquitoes attracted to person P_k was estimated by the following equation:

$$P_{ijk} = \frac{E_i + T_j + P_k}{\sum_l \exp\left(E_i + T_j + P_k\right)}$$

Parameter estimates provided an index of attractiveness for each person. Relatively higher or lower estimates corresponded to high or low degrees of attractiveness, respectively. Parameter estimates were calculated using person P_1 as the reference standard. Pairwise t-probabilities were calculated to establish significance levels of differences in mosquito catches between persons and ranks of attractiveness were assigned based on this criterion. Data were analysed using the General statistical computer software programme [36] (Genstat® for windows, 5th Edition).

Ethical clearance

Informed consent was obtained from all nine human participants. The project was approved by the Kenya National Ethical Review Committee at the Kenya Medical Research Institute (protocol KEMRI/RES/7/3/1).

Results

The experiments were conducted between January 7 and March 23, 2000. The temperature and relative humidity within the experimental set-up, measured for different purposes after the study, ranged between 21.93 – 27.72°C and 49.65 – 74.56%, respectively.

Parasite infection of volunteers

Microscopic examination of blood smears detected no malaria parasites in the participants' blood during the entire experimental period.

Mosquito response in the absence of human emanations

Experiments in which mosquitoes were released in the absence of humans demonstrated that the olfactometer was symmetrical, as entry responses did not differ significantly between traps ($P > 0.05$). The number of mosquitoes caught in trap A were not significantly different from those caught in trap B ($P = 0.808$) or trap C ($P = 0.147$). Of the mosquitoes released only 9.6% (49 of 512) were captured in the trap chambers (Table 1), the rest were either in the choice chamber or did not leave the release cup. The number of mosquitoes trapped during separate test periods varied from 0 to 9. There was no significant difference in the number of mosquitoes trapped during test period I and II ($P = 0.594$) or test period I and III ($P = 0.147$). There was also no significant interaction between test period and trap ($P = 0.138$).

Mosquito response in the presence of human emanations

Forty six percent (1688 of 3673) of the mosquitoes that were released during the experiments with the nine human subjects, three of whom were simultaneously present each time, were recaptured in the collecting cages. The rest were recaptured in the choice chamber or release cup. The experiments were conducted over 16 days encompassing a total of 48 test periods. The model with the best fit incorporated the factors person and trap and the interactions between (i) person and trap, (ii) person and test period and (iii) day and test period. The factor person significantly affected the number of mosquitoes caught per trap ($P < 0.001$). It was possible to classify the persons into high (P_1 and P_2), medium (P_4, P_5, P_7 and P_9) and low attractiveness (P_3, P_6 and P_8) groups. Parameter estimates for the persons are shown in Table 2. Although the behavioural responses of the mosquitoes were significantly affected by the factor trap ($P = 0.028$), there was no significant interaction between person and trap ($P = 0.302$) or person and test period ($P = 0.223$).

Response of mosquitoes to residual human odours

A significant effect of treatment was demonstrated when tents were occupied by one subject in each ($P < 0.001$). These differences were attributed to the factor person as participants did not shift between tents. P_2 attracted 2.7 and 5.6 times as many mosquitoes as P_5 ($P < 0.001$) and P_6 ($P < 0.001$), respectively. The effect of trap was not significant in the subsequent test period when the tents were empty ($P = 0.949$). Trap A had 1.1 and 1.0 times as many mosquitoes as trap B ($P = 0.869$) and C ($P = 0.873$), respectively. Mosquito catches in the presence and absence of human subjects are shown in Table 3.

Discussion

The tent olfactometer demonstrated its ability to discern differences in human attractiveness to mosquitoes. The system barred the potential interference of host irritability and defensive behaviour on mosquito responses. Interestingly, the mosquitoes preferred certain individuals despite being presented with emanations of three persons simul-

Table 1: The number of mosquitoes caught per trap in the absence of host-seeking stimuli. N, number of replicates. n, total number of mosquitoes trapped per test period also expressed (in parentheses) as the proportion of the total number of mosquitoes released.

Test period	N	Mean number trapped			n (proportion)
		Trap A	Trap B	Trap C	
20.00 – 21.00	2	8	9	3	20 (0.12)[a]
22.00 – 23.00	2	6	8	6	20 (0.12)[a]
24.00 – 01.00	2	3	1	5	9 (0.05)[a]
Total	6	20 (3.33)[a]	20 (3.33)[a]	14 (2.33)[a]	49 (0.096)

Numbers followed by the same letter (between traps or test periods) are not significantly different ($P > 0.05$).

Table 2: The mean number of mosquitoes attracted to each of nine Kenyan males and parameter estimates calculated for each individual person from the log-linear model. Calculations used person P_1 as the reference standard. Ranks of attractiveness are based on the level of significance of differences in the number mosquitoes attracted by pairs of persons. N, number of replicates. s.e., standard error of the mean.

Person	N	Estimate (β)	Mean catch ± s.e.	Rank of attractiveness
P_1	21	0	20.14 ± 3.17 a	1
P_2	15	0.030	18.20 ± 3.65 a	1
P_4	21	- 0.667	11.95 ± 1.83 b	2
P_5	21	- 0.651	11.33 ± 1.72 b	2
P_7	12	- 0.843	9.92 ± 1.55 b	2
P_9	9	- 0.711	9.67 ± 3.54 b	2
P_8	18	- 1.155	6.78 ± 1.01 c	3
P_6	15	- 1.204	6.73 ± 1.41 c	3
P_3	12	- 1.193	6.17 ± 1.12 c	3

Means not followed by the same letter are significantly different ($P < 0.05$).

taneously. This demonstrates the great discriminatory power exhibited by the mosquitoes in finding their blood-meal hosts. This capacity may be intrinsic as *An. gambiae* can bite selectively in settings where mixing of attractant stimuli is inevitable e.g. inside shared bedrooms or houses [8,9,11]. Although the evolutionary basis for this selective biting remains unknown, we have since not been able to demonstrate a positive correlation between fecundity and human attractiveness to the mosquitoes, i.e. feeding on highly attractive individuals did not yield larger batches of eggs than feeding on individuals with low attractiveness (WRM and BGJK, unpublished data). However, it has recently been suggested that blood-feeding insects may preferentially bite individuals whose cues signal less defensiveness [4].

In general, although it remains unknown why the mosquitoes were attracted more readily by certain individuals, it is certain that they did so in response to factors present in the person's total body emanations, which included odour, heat and moisture. Variability in the attraction of black flies [37] and the mosquitoes *An. gambiae* and *An. funestus* [31] to body emanations has been attributed to differences in carbon dioxide output rates. Carbon dioxide is thought to elicit take-off or sustain flight [38] in the short- and medium-range phases of host location [39]. However, carbon dioxide may not serve as a good kairomone for *An. gambiae* which, being highly anthropophilic, may have evolved a mechanism for distinguishing between individual humans using species-specific cues rather than doing so in response to differences in the level of exhaled carbon dioxide, which is not a human-specific kairomone [40]. In fact, *An. gambiae* is preferentially attracted to human body odour in disfavour of carbon dioxide [26]. In wind tunnel studies, carbon dioxide has been

Table 3: The number of mosquitoes caught per trap in the presence (test period I, 20.00 – 20.45 hours) and absence (test period II, 21.30 – 22.15 hours) of host-seeking stimuli. Traps A, B and C were linked to tents occupied by persons P_2, P_6 and P_5, respectively. n, the total number of mosquitoes trapped per test period also expressed (in parentheses) as a proportion of the total number of mosquitoes released.

Day	Test Period	Number of mosquitoes trapped			n (proportion)
		Trap A	Trap B	Trap C	
1	I	36	10	12	58 (0.55)
	II	2	4	2	8 (0.09)
2	I	33	3	8	44 (0.49)
	II	8	8	8	24 (0.25)
3	I	49	9	14	72 (0.72)
	II	5	2	5	12 (0.13)
4	I	27	4	20	51 (0.53)
	II	4	4	5	13 (0.13)
Total	I	145 (0.64)[a]	26 (0.12)[b]	54 (0.24)[c]	225 (0.57)
	II	20 (0.34)[a]	18 (0.31)[a]	20 (0.34)[a]	58 (0.15)

Totals not followed by the same letter in the same row are significantly different ($P < 0.001$).

shown to cause an inhibitory or neutral effect on the behaviour of *An. gambiae* [41,42].

The cues that impact greatly on mosquito orientation in the close vicinity of the host comprise body heat and moisture [39]. These factors may explain the differences in human attractiveness currently reported as mosquitoes were released ~1 m downwind of the participants inside tents. Black flies tend to bite man at rates that are partially related to inter-individual variation between skin and ambient temperatures [43]. Nonetheless, the role of olfaction in the short-range attraction of mosquitoes cannot be discounted [24] and is presumed to be caused by skin odour.

Even if residual human effluvia may have been left behind after participants exited the tents, such cues did not elicit mosquito behavioural responses. This result corroborates that of Braks [44] who found no preferential attraction of *An. gambiae s.l.* to an air stream exhausted from a tent containing unwashed clothing and recently used bedding material. Although there has been one report of higher catches of *An. gambiae s.l.* in huts containing worn clothing as opposed to empty ones [46], there are no other field reports of attraction of *An. gambiae* to residual stimuli adsorbed onto a holding material. Accordingly, Braks [44] suggested that the kairomones that induce behavioural responses in *An. gambiae* may be highly volatile and produced continuously from a living host but lost rapidly from worn clothing. More recently, Braks *et al*. [46] reported that the residual effect of highly attractive human sweat was lost within 20 minutes after release.

It remains interesting to note though that in our set-up (Table 3) sometimes equal or more mosquitoes were caught in tent B and C during the second (no human emanations present) than in the first (human emanations present) test periods. This shows that the number of mosquitoes caught by the most attractive individual had an effect on those caught by the other participants. However, the absence of significant differences in the number of mosquitoes caught between traps in the second test period implies that there were no individual-specific residual odour effects on the number of mosquitoes caught between tents.

The olfactometer developed in the current study can be used in various ways in studies of insect behaviour. First, the system can be manipulated so as to separate between components of total body emanations in order to study the effect of major fractions on insect behaviour. Second, as differences in attractiveness quantified based on man-landing catches (MLC) apparently resemble those measured using odour-baited entry traps [31], the set up may serve to replace MLC as its working principle differs less from that of OBETs [32]. However, care needs to be taken as the degree of attractiveness could differ depending on whether measurements are based on cues involved in short- or long-range orientation of host-seeking mosquitoes [33]. For instance, differential attractiveness of humans to black flies has been shown to vary when quantified using short- and long-range cues [37,43]. Third, the olfactometer offers a unique opportunity where effects of parasite infection and/or specific physical conditions of humans (e.g. pregnancy) on attractiveness can be examined with minimal ethical concerns. Fourth, the olfactometer offers the possibility to trap volatiles from individuals while at the same time conducting behavioural assays. In this way chemical bases for differential attractiveness of humans to mosquitoes can be explored more confidently.

Determination of reasons for variability in human attractiveness to mosquitoes could have several applications in terms of malaria control. First, once the chemical basis for increased attractiveness of humans to mosquitoes is understood it may be possible to identify and selectively protect those individuals most at risk of becoming infected as a consequence of being highly attractive to host-seeking mosquitoes. Second, development of an odour-baited trap that utilises human-specific infochemicals has the potential advantage of sampling mosquitoes that are reflective of the true host-seeking population. Continuous deployment of such traps for mosquito surveillance may lead to accurate disease forecasts thus enabling a more precise estimation of important parameters, such as entomological inoculation rates, without necessarily putting humans at the risk of infection as happens when mosquitoes are sampled by MLC. Third, it might be possible to manipulate the semiochemicals identified for use as lures in mass trapping as a means of vector control [47]. Fourth, it is thought that the attractant compounds identified may be applicable on hosts not commonly chosen in order to deviate host-seeking mosquitoes and reduce biting on humans [33].

Conclusions

The results of this study show that 1) variability in attractiveness of humans to *An. gambiae* exists and can be attributed to differences in the composition of complete body emanations encompassing body odour, heat and moisture, 2) residual odours do not affect behavioural responses and 3) the olfactometer developed can be used to unravel the evolutionary basis and chemo-ecological component of this phenomenon. Although sampling devices that utilise whole body emanations have been developed [32,48] the aim to develop odour-baited traps for mosquito control will remain unachieved until key factors associated with increased attractiveness to host-seeking mosquitoes have been identified and harnessed for use in trapping devices. Identification of such factors may contribute to the development of strategies to control malaria vectors through an integrated approach and thus augment on the potential impact of this approach in lowering entomologic inoculation rates in disease-endemic areas [49].

Authors' contributions

WRM designed the olfactometer, conducted all the experimental work, and drafted the original manuscript. RC assisted with statistical data analysis. BGJK conceived of the study, obtained funding for it, and supervised the experimental work and drafting of the final version of the paper in collaboration with WRM and WT.

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